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**Bacterial microbiota composition of a common ectoparasite of cavity-breeding birds, the Hen  
Flea *Ceratophyllus gallinae***

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Hen fleas *Ceratophyllus gallinae* are common haematophagous ectoparasites of cavity-breeding birds. Experimental field studies have demonstrated negative fitness consequences of hen flea infestations for bird hosts. Yet it is currently unclear if these negative effects are a direct consequence of flea-induced blood loss or a result of flea-borne pathogen transmission. Here we used a 16S rRNA sequencing approach to characterise the bacterial microbiota community of hen fleas collected from Great Tit *Parus major* nests. We found that *Staphylococcus* (Firmicutes), *Brevibacterium* (Actinobacteria), *Stenotrophomonas* (Proteobacteria), and *Massilia* (Proteobacteria) and as well as the arthropod endosymbionts *Candidatus* Lariskella and *Candidatus* Midichloria were most abundant. We found evidence for the occurrence of *Staphylococcus* spp. in fleas, which may cause opportunistic infections in bird hosts, but not of known pathogens commonly transmitted by other flea species, such as *Bartonella* spp. or *Rickettsia* spp. However, the pathogenic potential of many bacterial groups observed in our study is currently unknown. Furthermore, hen fleas might transmit other pathogens (e.g. viruses) which might contribute to the negative fitness consequences of hen flea infestations for their bird hosts in addition to the direct blood loss or secondary infections of wounds caused by biting fleas.

**Keywords:** host-parasite interactions, zoonotic diseases, nestboxes, garden birds, next generation sequencing (NGS), microbiome, pathogens, public health, wildlife disease

The hen flea *Ceratophyllus gallinae* is a common, generalist ectoparasite of cavity- and semi-cavity breeding birds of the Western Palearctic, such as tits (Paridae), sparrows (Passeridae) or flycatchers (Muscicapidae) (Tripet & Richner 1997). They live and reproduce in the nesting material and suck blood from nestlings and adults (Tripet & Richner 1997). Experimental manipulations of hen flea load in great tit nests demonstrated substantial negative fitness consequences of flea infestations for the bird host (e.g. Tschirren *et al.* 2003, Fitze *et al.* 2004). Yet, it is currently unknown whether these negative effects are caused directly by the ectoparasite (i.e. through blood loss) or indirectly through the transmission of flea-borne pathogens.

Many members of the flea order Siphonaptera are known vectors for wildlife, pet and / or human pathogens (Bitam *et al.* 2010). In total, there are over 2500 described flea species; the majority parasitises mammals, but about 6% are ornithophilic (Bitam *et al.* 2010). The most famous example of a flea-transmitted pathogen is *Yersinia pestis*, the causative agent of bubonic plague, which is vectored by a range of mammal flea species, including the Rat Flea *Xenopsylla cheopis* (Stenseth *et al.* 2008). Other pathogens transmitted by fleas include *Rickettsia* spp., the causative agents of diseases such as murine typhus (Traub 1978) and spotted fever (Pérez-Osorio *et al.* 2008), *Bartonella* spp., the causative agents of diseases such as cat-scratch disease and endocarditis (Chomel *et al.* 2006) or *Coxiella* spp., the causative agents of diseases such as Q fever (Angelakis *et al.* 2014). The vector potential of bird fleas, on the other hand, is largely unknown. However, a previous study on *Ceratophyllus garei* fleas infesting migratory reed warblers and their nests in Slovakia observed a *Rickettsia* spp. prevalence of 31.9% (Sekeyova *et al.* 2012), indicating that also bird fleas can act as vectors. Furthermore, evidence of *Bartonella* spp. infections was found in a range of bird species (Mascarelli *et al.* 2014).

Here we characterise for the first time the bacterial microbiota of *Ceratophyllus gallinae* fleas and survey the occurrence of known pathogens, for which hen fleas may act as a vector and which contribute to the lower fitness observed in hen flea-infested birds.

Understanding the vector-potential of hen fleas is not only important for our understanding of the eco-evolutionary dynamics of bird-ectoparasite interactions, but also for the identification of potential human health risks, as people are exposed to and bitten by hen fleas on a regular basis when cleaning out nest boxes in their garden (du Feu 1987).

## *Methods and materials*

### *Flea sampling*

We collected Hen Fleas *Ceratophyllus gallinae* from 22 nests of Great Tits *Parus major* breeding in nestboxes in Zurichbergwald, Switzerland (47°20'08"N, 8°30'01"E) in summer 2016. Fleas were stored in 95% ethanol.

### *Bacterial microbiota sequencing*

DNA isolation was performed in a laminar flow cabinet. Individual fleas ( $n = 208$  fleas from 22 nests; Table 1) were washed thrice with sterile water, once with 3% hydrogen peroxide and then crushed with a sterile pestle. Fleas from 7-8 nests were then pooled (Table 1) for DNA extraction and 16S sequencing. Nests were randomly allocated to pools. Whole-pool DNA extraction was performed using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

We quantified the bacterial microbiota of hen fleas by sequencing the hypervariable V3-V4 region of the 16S rRNA gene. The protocol has been described in detail elsewhere (Aivelo *et al.* 2019). In short, we prepared sequencing libraries following the Earth Microbiome 16S Illumina protocol using the primers 515F and 806R (Apprill *et al.* 2015, Parada *et al.* 2016), with an expected amplicon size of approximately 300 bp. The 16S region was amplified in a first PCR reaction, the PCR products were visualized on an agarose gel, cut out and purified with MinElute Gel Extraction kit (Qiagen, Hilden, Germany). Illumina Miseq adaptors were then added in second PCR step and

the products were purified as described above. 4 nM library pools were then created by mixing equimolar amounts of products, followed by standard normalization protocols. Paired-end sequencing of flea pools and negative controls ( $n = 5$ ) was performed on a Illumina MiSeq at the Functional Genomic Center Zurich using reagent kit 600cycle v3 (Illumina, San Diego, CA, USA) following the manufacturer's protocol, with a target amplicon length of 250 bp. Overall, we obtained 918 - 23296 amplicons across flea pools. We analyzed the sequence data with *mothur* pipeline (Schloss *et al.* 2009), following MiSeq standard operation procedures (Kozich *et al.* 2013). For sequence alignment, we used aligned SILVA bacterial references (release 132; <https://www.arbsilva.de/documentation/release-132/>) and grouped operational taxonomic units (OTUs) based on 99% sequence similarity. OTUs were assigned to taxa with the *wang* method (Wang *et al.* 2007) using SILVA taxonomy. We used taxonomic lists in Bitam *et al.* (2010), McElroy *et al.* (2010) and Eisen & Gage (2011) to identify flea-borne pathogens among the OTUs. Endosymbionts were identified by a literature search that combined the taxonomic labels and 'endosymbiont' as search terms in Web of Science (Clarivate Analytics, Philadelphia, PA, USA), following Aivelo *et al.* (2019). Sequences have been deposited to the Sequence Read Archive under BioProject PRJNA528393. Samples were rarefied to lowest amplicon count (1017) to account for amplicon number variation across pools. All OTUs with less than three amplicons across pools were discarded; these OTUs might represent sequencing errors or very rare bacteria that are unlikely to have major effects on host populations. Rarefaction did not alter the OTU composition of the more deeply sequenced pools 1 and 2. Indeed, Bray-Curtis dissimilarities between the non-rarefied and rarefied pool 1 was 0.14, and 0.09 in pool 2. The main effect of rarefaction was the removal of rare OTUs (from 954 to 58 in pool 1 and from 463 to 68 in pool 2). To compare the composition of the pools, we calculated alpha diversity (Inverse Simpson Index (Simpson 1949)) and beta diversity (Bray-Curtis dissimilarity (Bray & Curtis 1957) and UniFrac distance (Lozupone *et al.* 2011)) indices by using *vegan* (Oksanen *et al.* 2013) package in R (R Core Team 2013).

## Results

After filtering, 95 bacterial OTUs were identified across the three flea pools (mean: 66, range 58–73; Suppl. Table 1). 28 OTUs were observed in all three pools and 64 OTUs in two of the three pools (Suppl. Table 1). Inverse Simpson index (range: 7.0-11.8; Table 1), Bray-Curtis dissimilarity index (range: 0.49-0.64; Suppl. Table 2) and weighted UniFrac distances (range: 0.39-0.45; Suppl. Table 2) between pools were comparatively uniform, suggesting that there is a moderate level of dissimilarity among pools but none of the pools is markedly different in composition.

Most of the amplicons (97.7%) belonged to three bacterial phyla: Actinobacteria (24.0%), Firmicutes (29.2%) and Proteobacteria (44.4%)(Fig. 1; Suppl. Table 1). The most common OTUs were *Staphylococcus* (Firmicutes), *Brevibacterium* (Actinobacteria), *Massilia* (Proteobacteria), *Stenotrophomonas* (Proteobacteria), *Sporosarcina* (Firmicutes) and (Fig. 1; Suppl. Table 1).

No known bird or mammal pathogens were detected in the sequence data.

However, we found evidence for the occurrence of the arthropod endosymbionts *Candidatus* Lariskella, *Candidatus* Midichloria (both among the 6 most common OTUs) and *Rickettsiella* in hen fleas. No evidence for the occurrence of other arthropod endosymbionts (such as *Wolbachia* spp. or *Candidatus* Cardinium) was found.

## Discussion

We characterised the bacterial microbiota of the hen flea *C. gallinae*, a common, nest-based ectoparasite of cavity-nesting birds, using a 16S sequencing approach. In total, we observed 95 different OTUs across sequencing pools, which is broadly in line with the results of previous

mammal flea microbiota studies (Pornwiroon *et al.* 2007, Erickson *et al.* 2009, Jones *et al.* 2010, 2015). The phylum Proteobacteria as well as specific bacterial genera, such as *Brevibacterium* and *Stenotrophomonas*, were particularly abundant in hen fleas. Interestingly, these groups have also been found to dominate the bacterial communities of mammal flea species (Pornwiroon *et al.* 2007, Erickson *et al.* 2009, Jones *et al.* 2010).

No evidence for the occurrence of flea-transmitted bacterial (bird / mammal) pathogens such as *Bartonella* spp. or *Rickettsia* spp. (Bitam *et al.* 2010, McElroy *et al.* 2010, Eisen & Gage 2011) was found in our study. This contrasts the findings of a study in another bird flea, *C. garei*, which observed a high (31%) prevalence of *Rickettsia* spp. (Sekeyova *et al.* 2012). Currently it is unclear if this difference is due to spatial variation in *Rickettsia* spp. prevalence (i.e. Slovakia vs Switzerland) or if *C. gallinae* is a non-competent vector. Although studies testing for *Rickettsia* spp. infections in wild birds are still rare, there is some evidence that infections can occur, but that prevalence is low (Elfving *et al.* 2010). Similarly, evidence of *Bartonella* spp. infections has previously been found in several bird species (Mascarelli *et al.* 2014).

*Staphylococcus* spp., which can cause opportunistic infections (Coates *et al.* 2014), was another particularly common OTUs observed in hen fleas. Such opportunistic infections (e.g. of wounds caused by biting fleas) caused by *Staphylococcus* spp. or other opportunistic pathogens may contribute to the negative fitness effects of hen flea infestations on bird hosts (e.g. Tschirren *et al.* 2003, Fitze *et al.* 2004). Other particularly common OTUs across flea pools were arthropod endosymbionts *Candidatus\_Lariskella* and *Candidatus\_Midichloria*. Interestingly, however, we found no evidence for the occurrence of the endosymbiont *Wolbachia* spp., which is observed in many flea species (Gorham *et al.* 2006, Jones *et al.* 2015, Lawrence *et al.* 2015; but see Jones *et al.* 2010), including the bird flea *C. garei* (Sekeyova *et al.* 2012). It is possible that *Wolbachia* spp. is not truly absent in *C. gallinae* but was simply not detected because of low prevalence (only 208 fleas were sequenced in this study). Alternatively, negative interactions among endosymbionts (i.e.



competition effects; Aivelo *et al.* 2019) might explain the absence of *Wolbachia* spp. Indeed, a comparative survey of bacterial microbiota of mammalian fleas suggested that co-occurrence of endosymbionts (such as *Ca. Lariskella*, *Wolbachia* spp., *Spiroplasma* spp. and *Ca. Cardinium*) within fleas is rare (Jones *et al.* 2015). Thus, the comparably high *Ca. Lariskella* abundance in hen fleas may impede *Wolbachia* spp. infections.

Although only three pools of hen fleas were sequenced in our study, they included more than 200 fleas from 22 different great tit nests. Importantly, despite marked among-pool differences for some OTUs, overall the three independent pools showed remarkable similarities in bacterial microbiota composition, with the range of OTU diversity being small and almost half of the OTUs being present in at least two pools. Furthermore, a comparison of non-rarefied and rarefied pools highlighted that the lower sequencing depth of pool 3 did not bias the results. Together, it suggests that the bacterial communities we describe here for *C. gallinae* are representative.

As mentioned above, for some OTU's there were marked among-pool differences, most prominently for *Massilia* (Proteobacteria), which was very common in pool 1 but rare or absent in the other pools. These differences are likely due to environmental effects (e.g. differences in the composition of the nesting material among nests), which may affect some members of the flea microbiota. Indeed it is typically found that a part of the arthropod microbiota is non-resident (i.e. consists of bacteria that are obtained from the environment) and thus susceptible to environmental influences (Hammer *et al.* 2017).

Our study has a number of limitations. First, although we did not detect any known bacterial bird or mammal pathogens in our study, we cannot exclude the possibility that hen fleas may act as a vector for them, but that they were not detected because of very low prevalence. Also, for most OTUs observed in our study the pathogenic potential is currently unknown. Furthermore, only hen fleas from great tit nests were analysed and it is possible that hen flea microbiota composition might

differ depending on host species. Finally, we cannot exclude the possibility that there is spatiotemporal variation in bacterial microbiota composition of hen fleas, or in the occurrence of bacterial pathogens. The characterization of seasonal changes in the microbiota composition of hen fleas feeding on different hosts, as well as variation in hen flea microbiota composition on a large spatial scale would thus be an interesting next step.

In conclusion, we found no evidence that *C. gallinae* fleas harbour known bacterial bird (or mammal) pathogens, such as *Rickettsia* spp. or *Bartonella* spp. (Bitam *et al.* 2010, McElroy *et al.* 2010, Eisen & Gage 2011). The negative fitness consequences of hen flea infestations for their bird hosts (Tschirren *et al.* 2003, Fitze *et al.* 2004) are thus most likely caused by blood loss, secondary infections of wounds caused by biting fleas and / or other flea-borne pathogens (such as viruses or bacteria that are not currently recognised as bird pathogens).

The lack of (known) human bacterial pathogens in hen fleas furthermore suggests that the human health risk of exposure to hen fleas during nest box cleaning is likely limited. However, the pathogenic potential of most OTUs identified in our study is currently unknown and hen fleas may act as vectors for non-bacterial pathogens. Thus, to prevent exposure to such unidentified flea-borne pathogens, as well as a bad itch, we recommend to clean out nest boxes during a cold winter day when hen fleas are inactive.

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Table 1: Number of nests and individual hen fleas included in the sequencing pools and measures of bacterial microbiota diversity in the three pools.

Pool	Number of nests	Number of fleas	Fleas per nest (mean (range))	Number of OTUs	Alpha diversity (Inverse Simpson index)
1	8	68	8.5 (4-11)	58	7.0
2	7	70	10 (2-17)	68	11.8
3	7	70	10 (3-18)	73	10.8



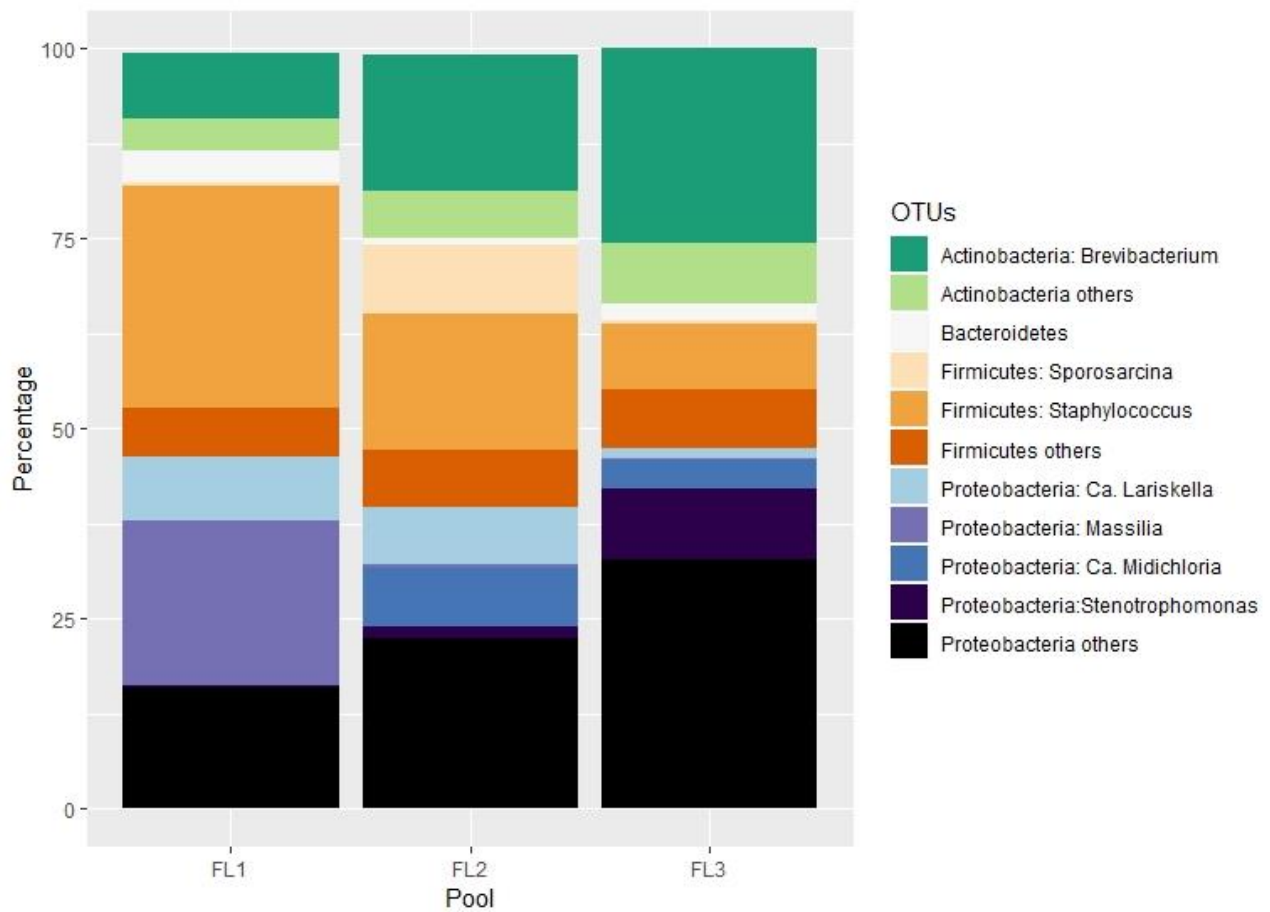


Figure 1: Phylotypic distribution of bacterial OTUs in hen fleas (pools FL1-FL3). The most common OTUs are shown separately whereas less common OTUs are grouped by phyla.